Statement of Problem Studied

The mechanisms and functions of a new class of biological materials discovered through self-assembly of ionic self-complementary oligopeptides was investigated. Three types of peptides have been classified. The type I peptides undergo intermolecular self-assembly. These self-assembing peptides have alternating ionic hydrophilic and hydrophobic amino acid residues. The side chains of these oligopeptides consist of repetitive positively charged residues arginine and lysine, and negatively charged residues glutamate and aspartate on the hydrophilic surface and alanines on the hydrophobic surface. A defining characteristic of this oligopeptide class is that charged side chains can form complementary ionic bonds. In most cases, these oligopeptides form exceedingly stable β-sheets in water.

These oligopeptides have been classified into several modulus, i.e. modulus I, II, III, IV, etc., and mixed moduli. Classification was based on the ionic surface of the molecules which have alternating + and - charged amino acid residues, alternating by 1, 2, 3, 4 and so on. For example, molecules of modulus I have the ionic arrangement - + - + - + - +, of modulus II - - + + - - + +, and modulus IV - - - - + + + + or the reversed charge orientation. It appears that the composition of EAK and RAE is more stable than those of DAK and RAD of the identical length. Systematic analysis also shows that modulus I peptides $(- +)_n$ are somewhat less stable than those of modulus II peptides $(- - + +)_n$. These ionic β-sheet oligopeptides can undergo self-assembly to form macroscopic biological materials and can be readily stained with Congo Red. Such biological materials represent a new generation of peptide-based biomaterials and can be further developed as scaffolding for tissue engineering. Type II peptides undergo both intermolecular and intramolecular self-assembly. When the peptides are in the β-sheet structure, they undergo intermolecular self-assembly. When the β-sheet lattice is disrupted by heat or change of pH, these peptides undergo intramolecular self-assembly to form stable monomeric helices. Type III peptides undergo surface self-assembly to form monolayers, thereby modifying their surface properties.

Table 1. Self-assembling oligopeptides studied.

Name	Sequence (n-->c)	Modulus	Structure
	+ - + - + - + -		
RADA16-I	n-RADARADARADARADA-c	I	β
	+ - + - + - + -		
RGDA16-I	n-RADARGDARADARGDA-c	I	r.c.
	+ - + -		
RADA8-I	n-RADARADA-c	I	r.c.
	+ + - - + + - -		
RAD16-II	n-RARADADARARADADA-c	II	β
	+ + - -		
RAD8-II	n-RARADADA-c	II	r.c.
	- + - + - + - +		
EAKA16-I	n-AEAKAEAKAEAKAEAK-c	I	β
	- + - +		
EAKA8-I	n-AEAKAEAK-c	I	r.c.
	+ - + - + - + -		
RAEA16-I	n-RAEARAEARAEARAEA-c	I	β
	+ - + -		
RAEA8-I	n-RAEARAEA-c	I	r.c.
	+ - + - + - + -		
KADA16-I	n-KADAKADAKADAKADA-c	I	β
	+ - + -		
KADA8-I	n-KADAKADA-c	I	r.c.
	- - + + - - + +		
EAH16-II	n-AEAEAHAHAEAEAHAH-c	II	β
	- - + +		
EAH8-II	n-AEAEAHAH-c	II	r.c.
	- - + + - - + +		
EFK16-II	n-FEFEFKFKFEFEFKFK-c	II	β
	- + - +		
EFK8-II	n-FEFKFEFK-c	I	ND
	- - + + - - + +		
ELK16-II	n-LELELKLKLELELKLK-c	II	ND
	- - + +		
ELK8-II	n-LELELKLK-c	II	r.c.

	- - + + - - + +		
EAK16-II	n-AEAEAKAKAEAEAKAK-c	II	β
	- - - - + +		
EAK12	n-AEAEAEAEAKAK-c	IV/II	α/β
	- - + +		
EAK8-II	n-AEAEAKAK-c	II	r.c.
	+ + + + - - - -		
KAE16-IV	n-KAKAKAKAEAEAEAEA-c	IV	β
	- - - - + + + +		
EAK16-IV	n-AEAEAEAEAKAKAKAK-c	IV	β
	+ + + + - - - -		
RAD16-IV	n-RARARARADADADADA-c	IV	β
	- - - - + + + +		
DAR16-IV	n-ADADADADARARARAR-c	IV	α/β
	- - - - + + + +		
DAR16-IV*	n-DADADADARARARARA-c	IV	α/β
	- - - - + + + +		
DAR32-IV	n-(ADADADADARARARAR)-c	IV	α/β
	+-+-+++++-+-++++		
EHK16	n-HEHEHKHKHEHEHKHK-c	N/A	r.c.
	+-+-++++		
EHK8-I	n-HEHEHKHK-c	N/A	r.c.
	- - - - - - - - - -		
VE20*	n-VEVEVEVEVEVEVEVEVE-c	N/A	β (NaCl)
	+ + + + + + + + + +		
RF20*	n-RFRFRFRFRFRFRFRFRF-c	N/A	β (NaCl)

β, β-sheet; α, α-helix; r.c., random coil; N/A not applicable; ND, not determined. *Both VE20 and RF20 are in β-sheet form when they are incubated in solution containing NaCl. They do not self-assemble to form macroscopic matrices. N/A, not applicable.

Summary of Important Results

A. Self-Complementary Oligopeptide-Matrices Support Mammalian Cell Attachment

A new class of ionic *self-complementary* oligopeptides has been developed. Two members of this class have been designated RAD16 and EAK16. These oligopeptides consist of regular repeats of alternating ionic hydrophilic and hydrophobic amino acids and associate to form stable β-sheet structures in water. The addition of buffers containing millimolar amounts of monovalent salts or the transfer of a peptide solution into physiological solutions results in the spontaneous assembly of the oligopeptides into a stable, macroscopic membranous matrix. The matrix is composed of ordered filaments which form porous enclosures. A variety of mammalian cell types are able to attach to both RAD16 and EAK16 membranous matrices. These matrices provide a novel experimental system for analyzing mechanisms of *in vitro* cell attachment and may have applications in *in vivo* studies of tissue regeneration, tissue transplantation and wound healing.

B. Extensive Neurite Outgrowth on Transportable Self-complementary Oligopeptide Matrices

We have developed a new class of peptide matrix biomaterials that spontaneously assemble from β-sheet ionic, self-complementary oligopeptides under physiological conditions. These matrices serve as a scaffold for cell attachment and support extensive neurite outgrowth. The matrices with cells attached and a network of neurites are readily transportable from one medium to another. A number of mammalian cells including primary mouse cerebellar granules, hippocampal neuronal cells and NGF-differentiated PC12 cells as well as human neuroblastoma cells have demonstrated their ability to attach to these matrices. It has also been shown that neuronal cells project extensive neurite growth, following the contour of the matrices across the surface up to 500 micrometers in two weeks. These peptide matrices have shown no apparent cytotoxicity and do not elicit a measurable immune response, inflammation or gliosis in animals. This system may be further developed as a biological scaffold

for nervous system repair in patients with spinal cord injuries and in tissue engineering.

C. Direct Structural Transformation of Ionic Oligopeptides: A New Class of Biological Molecular Switches

A subclass of ionic oligopeptides that undergoes drastic structural transformations from a β-sheet to an α-helix under stimuli of temperature and pH changes was investigated. These oligopeptides are derivatives of a new class of self-assembling ionic oligopeptides and have two distinctive surfaces with regular repeats of alternating ionic hydrophilic and hydrophobic side chains. A defining characteristic of this class of peptide molecular switches is that the cluster of positively charged arginine or lysine is located toward the C-terminus and the negatively charged glutamate and aspartate is located toward the N-terminus of the oligopeptide. Such an arrangement of sequences and charge clusters balances the α-helical dipole moment (C-terminus-->N-terminus) and promotes helical formation. The composition of both aspartate-alanine-arginine (DAR) and glutamate-alanine-lysine (EAK) are effective in acting as molecular switches in structural transformation. Changes of orientation in the charged clusters and single alterations or substitutions of charged residues block direct structural transition. These substitutions abolish the capability of peptide self-assembly, thus eliminating matrix formation. Size changes accompanying the structural changes are approximately twofold, and such changes are analogous to an on/off molecular switch. These findings could possibly be further developed for use in biological sensors and medical detection devices. Further development of this subclass of ionic oligopeptides could result in their possible use in coatings for metallic and polymer surfaces.

D. Self-assembling Oligopeptides on Solid Surfaces for Cell Adhesion and Nerve Fiber Outgrowth

The primary goal of this project is to develop alternative biological surface coating materials for cell adhesion, migration and neurite outgrowth. We have incorporated this class of self-assembling oligopeptides with defined size into unique biological ligands, specifically suitable for cell-material interaction. This class of self-assembling oligopeptide-based materials has several advantages over

existing materials. These include: 1) complete *de novo* design with high precision and multiple features, 2) peptide materials that can be chemically synthesized and HPLC purified and are free of contaminants, 3) surface coating that is a homogenous monolayer and can be precisely controlled, 4) producing defined materials that provide the opportunity to study detailed cell-material interactions, and 5) specific surface patterns that can be designed, fabricated and coated with self-assembling peptide materials. Several oligopeptides have been designed with cell adhesion motifs at one end and a unique amino acid-cysteine containing thio group on the other end so that they can be covalently linked to other surfaces. These peptides can be used to self-assemble on uniform gold surfaces of pre-patterned glass slides. These patterns are designed so that they have alternating tracks with cell adhesion peptides and non-cell adhesion materials. Preliminary results have demonstrated that the self-assembling peptides containing cell adhesion motifs are an excellent substrate for cell-material interaction. Several types of cells have already been tested, including primary cells. The cells have proved to be well spread, robust and healthy. PC12 cells have been differentiated on a peptide-coated surface with NGF. Using this well-controlled self-assembling oligopeptide system has a significant implication in the study of cell-material interactions and cell migration. Since these peptides can be systematically designed, a number of experiments have been planned to study their mechanical compliance and their behavior in cells.

E. Development of Self-assembling Oligopeptide Matrices for Tissue Engineering

The long-term objective of this project is to gain a fundamental understanding of tissue engineering using a new class of biological materials made through self-assembling ionic β-sheet RAD peptides that form a matrix scaffold. The immediate objective is to develop a class of peptide scaffolds as an artificial extracellular environment for cell attachment. A key challenge to further develop cell-based transplants for treatment neurological diseases is a transplantable system that is optimal for the target. This project stems from the hypothesis that a peptide-based scaffold is superior to existing biomaterials for tissue engineering. The peptide scaffold can be a) systematically designed based on the molecular structure governing self-assembly, b) processed in aqueous

solution and fabricated to a desired geometry, c) used as a scaffold to support cell attachment, proliferation and neurite outgrowth, and d) biocompatible, that is, a matrix that will not elicit inflammatory or immune reactions and also be biodegradable.

One of the most valuable features of this peptide-based scaffolding is that it is readily transportable from one medium to another with cells remaining attached and retaining their existing network of neurites without damaging the integrity of the cell-scaffold complex. Information gained from this study could be applied to other tissues and biological systems. These objectives will be accomplished in the following ways: a) development of ionic oligopeptide matrix scaffolding--molecular design, synthesis and structural characterization of a biocompatible, biodegradable scaffold and to systematically assess its capability for supporting nerve regeneration; b) testing cell-material interactions of this peptide-based scaffold with a variety of cells; and c) testing to determine if the peptide matrix scaffold is superior to existing scaffolds, e.g. collagen gels.

F. Production of Genetically Engineered Self-assembling Peptides in *E.coli* Cells and Germline Transgenic Plants

Two artificially designed genes have been cloned that encode self-assembling ionic peptides. These peptides are designed with a cell adhesion motif arginine-alanine-aspartate-serine [M(RADS)n]m (n=6, m>2) that can be used as a scaffold for tissue engineering. These genes were designed specifically for use in large-scale biological materials production. To that end, one of the genes has now been expressed in *E.coli* and can be purified as an artificial protein product. Another gene (RADS)6 has been cloned into a plasmid vector and will be transferred into a plant vector for generating stable germline transgenic plants. It is hoped that large-scale production of these peptide matrices in plants will drastically reduce the cost of biological materials in the future. This plant-based expression could eventually be used as a model system to be generally applied to other biomaterials as well as in the production of protein-based systems.

List of Publications

1. Shuguang Zhang, Todd Holmes, Michael DiPersio, Richard Hynes, Xing Su and Alexander Rich (1995) Self-complementary oligopeptide matrices support mammalian cell attachment. *Biomaterials* **16**, 1385-1393.

2. Shuguang Zhang (1996) Design and exploitation of self-assembling ionic complementary peptide systems: A model for peptide biomaterial engineering in *Perspective in Protein Engineering* 1996 (CD-ROM Edition) (Geisow, M. J., Ed.), Biodigm Ltd. (UK). (Net search key words: protein engineering). ISBN 0-9529015-0-1. **<http://www.biodigm.com/pope/cdrom3.htm>**.

3. Shuguang Zhang & Alexander Rich (1997) Direct conversion of an oligopeptide from a _-sheet to an _-helix:Amodel for amyloid formation. *Proc. Natl. Acad. Sci. USA* **94**, 23-28.

4. Shuguang Zhang, Michael Altman, Rodney Chan, Peter Lee & Richard Ma (1998) Self-assembling peptides in biology, materials science and engineering *Peptide Science: Present and Future.* (Ed. Shimonishi, Y.) Kluwer Publishers, Amsterdam 761-768.

5. Shuguang Zhang & Michael Altman (1998) Peptide self-assembly nanosystem in materials science and engineering. *The US Army Research Office Report* (**In press**).

6. Shuguang Zhang & Michael Altman (1998) Peptide self-assembly in functional polymer science and engineering. *Reactive and Functional Polymers* (**In press**).

7. Shuguang Zhang, Lin Yan, Michael Altman, Michael Lässle, Helen Nugent, Felice Frankel, Douglas A. Lauffenburger, George Whitesides, & Alexander Rich (1999) Biological surface engineering: A simple system for cell pattern formation *Biomaterials* (**In press**).

8. Todd Holmes, Sonsoles Delacalle, Xing Su, Alexander Rich and Shuguang Zhang (1997) Extensive neurite outgrowth on transportable self-complimentary oligopeptide matrices (**Submitted**).

9. Michael Altman, Peter Lee, Alexander Rich & Shuguang Zhang (1999) Secondary structure dynamics of ionic self-complementary peptides (**Submitted**).

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13. ABSTRACT *(Maximum 200 words)*

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